UNIVERSITY OF PENNSYLVANIA

EFFECTS OF CERTAIN ACIDS AND THEIR SODIUM SALTS UPON THE GROWTH OF SCLEROTINIA CINEREA

MARIN SHEPPARD DUNN

A THESIS

IN BOTANY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

PHILADELPHIA

1926

EFFECTS OF CERTAIN ACIDS AND THEIR SODIUM SALTS UPON THE GROWTH OF SCLEROTINIA CINEREA

A THESIS

IN BOTANY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF PENNSYLVANIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MARIN SHEPPARD DUNN

Reprint from
American Journal of Botany, Vol. XIII, No. 1

PHILADELPHIA

1926

R. UNIV.
BIBLIOTHEEK
LEIDEN

Reprinted from the AMERICAN JOURNAL OF BOTANY, XIII: 40–58, January, 1926.

EFFECTS OF CERTAIN ACIDS AND THEIR SODIUM SALTS UPON THE GROWTH OF *SCLEROTINIA CINEREA*

MARIN SHEPPARD DUNN

(Received for publication April 20, 1925)

INTRODUCTION

Various studies have been made of the toxic effects of certain acids upon diverse plant and animal material, and in some cases the investigators have tried to account for the toxic action observed. The results are somewhat conflicting. Wolf and Harris (18), working with *Bacillus perfringens* and *B. sporogenes*, came to the conclusion that hydrochloric, lactic, formic, butyric, acetic, malic, and succinic acids affect the growth of *B. perfringens* in proportion to the hydrogen-ion concentration. The anions and undissociated acids behave merely as foreign bodies possessing a certain inhibiting influence in proportion to their molecular concentration. From their studies of the action of formic acid and certain salts upon *B. typhosus*, Norton and Hsu (13) concluded that the disinfecting power of an acid is approximately proportional to the hydrogen-ion concentration. Krönig and Paul (10), as a result of their investigations on the effect of a series of compounds, organic and inorganic, upon *Bacillus anthracis*, state that there is a general relationship between the disinfecting power and the amount of dissociated hydrogen present with the acids employed. However, certain investigators find that the toxic action of certain acids may be attributed in large part to other causes than the hydrogen ion. Clark (2), who used the hanging-drop method to study the toxic effect of certain deleterious agents upon the germination and subsequent development of *Aspergillus flavus*, *Sterigmatocystis nigra*, *Oedocephalum albidum*, *Penicillium glaucum*, and *Botrytis vulgaris*, found that the toxic properties of acetic acid are to be attributed almost wholly to the undissociated molecule. Collett (4) worked with *Paramoecium caudatum*, *Stylonichia pustulata*, *Euplotes patella*, and *Vorticella nebulifera*. She observed that the hydrogen ion is not the only factor in toxicity, for, in solutions of equal pH, the acids used were not equally toxic. Depression of ionization showed that the anions of formic, acetic, propionic, butyric, valeric, citric, benzoic, phthalic, and salicylic acids are toxic to Paramoecium and Euplotes. She also found that mixtures of certain acids (formic, acetic, butyric, and valeric) with non-toxic concentrations of their salts are more toxic than can be accounted for by the pH of the mixtures. The same thing applied to salicylic acid when mixed with a dilute solution of the sodium salt. This seems to indicate that the molecules are in themselves toxic. Kahlenberg and True

(9) investigated the effect of certain acids upon the growth of the roots of seedlings of *Lupinus albus* L. Their results obtained with acetic and butyric acids show that the undissociated acid is toxic. In another paper, True (15) reported that in inorganic acids the purest H-ion effect is seen, while in those of the fatty acid series, which dissociate much less freely, the undissociated molecule plays an important rôle often exceeding that played by the H ions. In general, the anions of organic acids possess relatively slight toxic properties. Winslow and Lochridge (17) investigated the disinfecting power of hydrochloric, sulfuric, acetic, and benzoic acids upon the typhoid and colon bacilli. Their investigations led them to conclude that the toxicity of the two inorganic acids is due principally to the H ion. In the cases of acetic and benzoic acids, the toxic factor is apparently the anion or the undissociated molecule—the latter, being so much greater in amount, probably playing the principal part.

It is extremely difficult to draw definite conclusions concerning the relative effects of various acids on plants from much of the literature because of the varied material, the methods of study, and the absence of exact hydrogen-ion determinations, especially in the older writings. In general, however, there is indication that the toxic effects of the mineral acids hydrochloric and sulfuric are due chiefly to the hydrogen ion. In the case of the usual organic acids employed, the toxic action seems to be due to the combined influence of the H ion, the anion to a slight degree, and the undissociated molecule.

Another problem that is opened by the investigations of the toxicity of an acid is that of the limiting hydrogen-ion concentration for the growth of an organism. There are numerous references to these limits in the literature, and only a few will be cited here. Taylor (14), using four different kinds of bacteria, found indications that among the common organic acids there is a great variation or specificity in their activity toward the organisms used. Wolf and Harris (18) gave as the mean critical pH values for the seven acids used with *B. perfringens*, pH 4.82, and with *B. sporogenes*, pH 4.94. In their work on the roots of the seedlings of *Lupinus albus* L., Kahlenberg and True (9) found 1/6400 M per liter the limit for apparently normal growth with hydrochloric, phosphoric, sulfuric, formic, and salicylic acids. Acetic acid had a limit of 1/1600 M per liter and butyric acid one of 1/3200 M per liter. Much of the work has been conducted, however, using a nutrient solution to which varying amounts of some one acid were added. Johnson (8) found the following critical pH values, using hydrochloric acid: *Mucor glomerula*, pH 3.2–3.4; *Aspergillus terricola*, pH 1.6—1.8; *A. oryzae*, pH 1.6–1.8; *Penicillium variabile*, pH 1.6–1.8; *P. italicum*, pH 1.9–2.2; *Fusarium bullatum*, pH 2.0–2.2; and *F. oxysporium*, pH 1.8–2.2. Fred and Loomis (6) used a mannitol solution as a culture medium and adjusted its reaction by the addition of sterile N/10 sulfuric acid. There was no growth of *B. radicicola* when the pH of the culture medium was 2.7 or over. In

their study of the reaction of soil solutions, Morgan and Gruzit (12) found that an acid reaction of the soil solution adjusted by means of a mineral acid had a distinct toxic effect at N/1200 on the growth of bacteria.

It is the purpose of this investigation to determine whether the hydrogen ion is always the chief factor of toxicity in the effect of various acids upon the germination and growth of fungous spores, or, if not, to indicate a possible toxic factor or factors involved. It is thought that, at the same time, work of this nature will throw much light upon the limiting hydrogen-ion concentrations for the organism used when grown in the presence of certain acids, and will also show the changes in the condition of the nutrient solution by the reaction of the organism. For this purpose the following experiments have been planned, using six acids which have frequently been employed by various other investigators.

Methods

Organism. The organism employed in the following investigations was the brown-rot fungus, *Sclerotinia cinerea* (Bon.) Schröt. This fungus was chosen because of its ready fruiting and rapid growth, and was obtained from the Bureau of Plant Industry through the kindness of John A. Roberts. By means of the microloop (5), a colony was obtained from a single-spore inoculation of peach decoction. The spores used in the investigations recorded in this paper were all descendants of the same single spore.

Culture Solution. The culture solution used was a modified Richards' solution. *Sclerotinia cinerea* grew upon it excellently and produced abundant spores. The composition of the culture medium employed was as follows:

Extract of 100 g. of dried peaches per liter;
Cane sugar, 20 g. per liter;
Peptone, 2 g. per liter;
$Ca(NO_3)_2$ 0.5 M, 4 cc. per liter;
$MgSO_4$ 0.5 M, 2 cc. per liter;
KH_2PO_4 0.5 M, 4 cc. per liter;
Distilled water, 1000 cc.

Six hundred grams of dried peaches were cut up and boiled gently in two liters of water for two hours. The peach juice was decanted, and then the pulp remaining was reboiled for one hour with about one liter of water. The decanted juice was then added to the first decanted juice. Next the peaches were pressed in a fruit press, and then the assembled juice was filtered while hot through cotton and made up to three liters. The other ingredients were then added, and the solution was autoclaved for fifteen minutes at fourteen pounds. In order to lower the acidity of the solution, N/1 NaOH was added until the culture medium reached in most cases a pH of 5.2. Consequently, because of the varying amounts of sodium hydroxid it was necessary to add, the mean total volume of the stock culture solution was 3285 cc. $\pm$ 0.8 percent. That the presence of the sodium hydroxid had no appreciable deleterious effect is shown by observing the growth curves (text fig. 1).

Stock cultures of the fungus were grown on 3 percent peach-agar slants. Inoculations (except in one instance later mentioned) were always made with spores from eleven-day-old cultures which had grown on 3 percent peach-agar slants, care always being taken that the size of the tubes was the same and that the angle of slant was also the same. In this way, differences in age of spores and in the amount of available growing surface were avoided.

150-cc. Erlenmeyer flasks were used for fungus-growth. Into each one of these was placed 25 cc. of the nutrient solution of twice the concentration, and distilled water and the acid or salt to be studied were added in varying proportions as required to make a total volume in each flask of 50 cc. Each series was always run in duplicate and often in triplicate. The series of flasks containing the medium under consideration was made at the same time and always as near to the day of inoculation as possible. The flasks were tightly plugged with cotton and autoclaved for fifteen minutes at fourteen pounds.

A duplicate culture was made for each step in a particular series. These duplicate flasks, instead of being inoculated, were used for obtaining the initial hydrogen-ion concentration of the particular step in the series after autoclaving.

At the time of inoculation, 10 cc. of sterile distilled water was added to the inoculation tube containing the fungus growing on 3 percent peach agar. The culture tube was then carefully rotated between the hands for about three minutes. The water containing the detached spores was re-decanted into the sterile tube which had originally contained it, and was again rotated for three minutes to secure separation of the spore groups. A platinum loop, whose size was not changed during the various series, was used for inoculation. One loopful of the inoculum was added to each of the Erlenmeyer flasks. After the entire series had been inoculated, the flasks were placed in the same dark closet and the fungus was allowed to grow for ten days. The contents of the flasks was then filtered, and the filter papers and flasks were washed at least three times with distilled water. In certain cases, for each step in a series, the nutrient solution upon which the fungus had grown was filtered directly into clean flasks. In such cases, no additional water was allowed to run into the flasks by the washing of the filter paper. Four drops of toluol were added, the flasks were tightly plugged with cotton, and their pH was determined on the following day. After the flasks containing the solutions for hydrogen-ion concentration determinations had been removed from the filter rack, the filters were washed in the usual way. The filters were dried in an electric oven at 85°–95° C. for at least 24 hours, placed in a desiccator, allowed to cool, and weighed. As each filter paper had, previous to filtration, been heated in the electric oven at the same temperature for twelve hours and then placed in a desiccator and weighed when cool, it was necessary only to subtract the

dry weight of the filter from the weight of the filter plus the fungus to obtain the weight of the mycelium. Incubation was at laboratory temperature, which had a mean of about 22° C. although in one case the mean temperature reached 24.6° C. Never did the temperature go below 18° C., and only once was it recorded as high as 28° C. To obviate these differences in temperature, the results have been reported on a percentage basis with the control for each series taken as representing 100 per cent.

Care of Glassware. All glassware was cleaned by subjecting it twice to boiling potassium bichromate-sulfuric acid cleaning solution. It was then heated twice with boiling distilled water and rinsed two or three times in cold distilled water. Pipettes and other measuring vessels were always cleaned and standardized.

Chemicals. The acids and salts used in these experiments were the purest obtainable. Sulfuric acid, phosphoric acid, glacial acetic acid, and sodium dihydrogen phosphate. $2H_2O$ were Powers-Weightman-Rosengarten analytical chemicals. Formic acid 85 percent, butyric acid C.P., sodium formate C.P., and sodium salicylate C.P. were obtained from Coleman and Bell Company. Salicylic acid and sodium sulfate. $10H_2O$ came from Merck, sodium acetate C.P. from the J. T. Baker Company, and sodium butyrate (tech.) from the Eastman Kodak Company.

The pH values recorded in this paper were all obtained electrometrically by the use of a potentiometer. The colorimetric method was tried at first, using as standards McIlvaine's Na_2HPO_4-citric acid buffer solution (3) and adding the correct amounts of the appropriate indicators, brom phenol blue, brom cresol purple, thymol blue, etc. A comparison of the pH values derived from this method with those found electrometrically showed that the colorimetric method could not be used, as the error varied with different solutions. Undoubtedly, strong coloration and turbidity rendered any exact reading impossible.

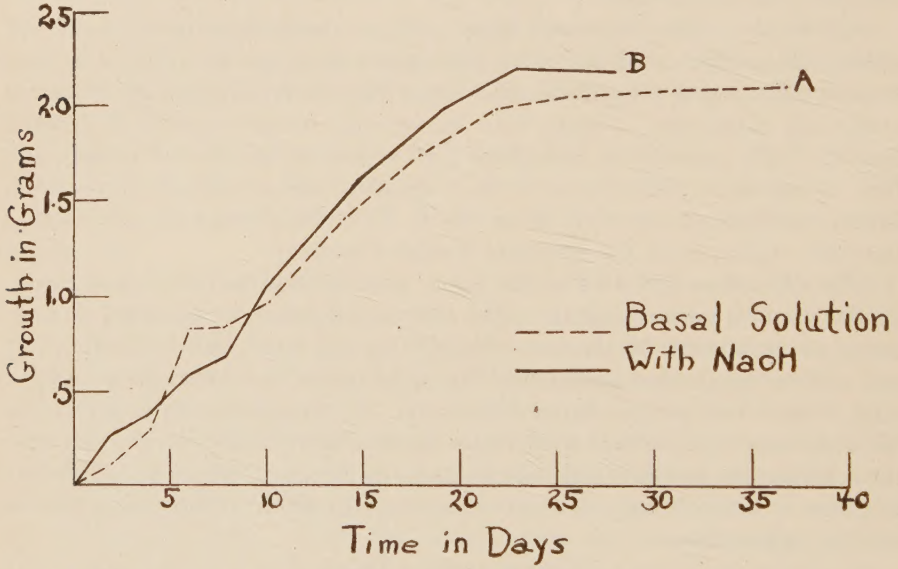
Experimental Data

Before studying the effects of the various acids and salts upon the growth of *Sclerotinia cinerea*, it was necessary to determine the growth of the fungus in the culture medium to which no N/1 sodium hydroxid had been added, and then to compare this result with the growth of the fungus in the medium which had had its hydrogen-ion concentration lowered by the addition of the necessary amount of N/1 sodium hydroxid to bring the pH to approximately 5.35. Changes in the hydrogen-ion concentration during this growth period were also obtained. Thirty flasks containing 25 cc. nutrient solution of double concentration plus 25 cc. distilled water were inoculated with spores from a ten-day-old peach-agar slant. The flasks were harvested, three flasks at a time, as shown by text figure 1. The dry weights were obtained in the usual manner, and the average weight was calculated. From the average weights, taken at various intervals, the curve *A* in text figure 1 was obtained. It was found that the growth of the

fungus during the first two days was very slow, but that the rate increased and became rapid at 10 days. Furthermore, the growth curve became flattened at about the 23d day.

The changes in hydrogen-ion concentrations of this series were followed colorimetrically from day to day, but the results were later discarded upon finding that the approximate values could not be accurately determined.

Another series was therefore undertaken in which the changes in hydrogen-ion concentration were recorded. These readings were accurately obtained electrometrically. It was found that, starting with pH 3.8, there was a slight increase of acidity followed by a decided decrease. On the 16th day, the pH was 4.25.



TEXT FIG. 1. The effect of the addition of NaOH to the basal nutrient solution upon the rate of growth of *Sclerotinia cinerea*.

Text figure 1, *B* shows the growth curve of the series in which the nutrient solution had its hydrogen-ion concentration lowered to pH 5.35 by the addition of N/1 sodium hydroxid. It may be seen to follow approximately curve *A*. The addition of sodium hydroxid had therefore no appreciable effect upon the growth of the fungus. In both cases, the temperature was approximately the same, 22°–25° C. The hydrogen-ion concentration of this third series showed a rise to pH 4.4 on the 6th day, followed by a fall to pH 5.65 on the 28th day.

Other cultures in other series during the course of these experiments have shown that the hydrogen-ion concentration is changed finally to pH 7 +. One culture gave pH 7.1 at the end of thirty-two days.

From these results the following conclusions were drawn: *Sclerotinia*

cinerea at first raises the hydrogen-ion concentration of the medium and later depresses it above the initial, finally changing it to pH 7.0 +. The addition of N/1 sodium hydroxid to lower the hydrogen-ion concentration has no appreciable deleterious effect under the conditions of the experiment. The fungus may be harvested at the end of 10 days' growth with the assurance that the growth rate has not decreased (text fig. 1).

The results of the various acid and salt series are shown in tables 1–17. Column 1 of each table gives the number of cc. of acid or salt used; column 2 the initial pH of the culture medium with the added acid or salt before inoculation; column 3, the percentage growth based on the control for the particular series; column 4, the final pH after the fungus had been growing on the culture medium for 10 days; column 5, the normality of the nutrient solution before autoclaving but after the addition of the proper amount of salt or acid. It is to be noted that in every case the average temperature is indicated at the head of the table.

A precipitate appeared in the sulfuric acid, phosphoric acid, and sodium phosphate cultures. Check cultures, not inoculated, were filtered, and the dry weight of the precipitate was obtained in the usual manner. This weight was then subtracted from the corresponding values of the weight of the fungus upon the filter paper.

Throughout the investigation there have been three principal causes for variation, and these must be considered in critically studying the tables. The hydrogen-ion concentration of the original peach solution varied slightly (pH 3.8, pH 3.9, pH 4.0, etc.) in the various series—thus necessitating the addition of varying quantities of N/1 sodium hydroxid. There were small differences in the growth temperature. A certain amount of each culture was lost through autoclaving and standing, although great care was taken in the use of the autoclave and the flasks were plugged as tightly as possible with cotton.

It may be seen from the tables that a slight increase in acidity is usually beneficial for growth of *Sclerotinia cinerea*. This is in harmony with the work of Webb (16), who found that *Botrytis cinerea* has the crest of its germination curve at pH 3.1. With sulfuric acid, the greatest amount of growth was obtained at pH 2.85 (table 1), with phosphoric acid at pH 3.90 (table 3), and with formic acid at pH 4.37 (table 5). One series with acetic acid gave 109 percent growth at pH 4.66 (table 7), while another series failed to give an increase above the control (table 8). In the case of butyric acid (tables 11, 12, 14) and salicylic acid (table 16), an increase in active acidity did not give an increase above the control for the series.

In almost every case, the original pH of the culture medium was changed by fungous growth. The final pH values differed for the various acids. When *Sclerotinia cinerea* was grown in the sulfuric acid solutions, the culture medium with an original acidity lower than pH 4.35 became more acid at the end of ten days. When it grew in solutions with initially high acidity,

Experimental Data Showing the Influence of the Addition of Certain Acids and Their Sodium Salts upon the Growth of Sclerotinia cinerea

TABLE 1

H_2SO_4(2N) Av. T. 23° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.25	100.0*	4.50	——
.3	4.35	94.2	4.35	.0120
.6	3.60	98.0	3.75	.0241
.8	3.20	103.0	3.55	.0321
1.0	2.85	118.3	3.15	.0400
1.2	2.53	70.9	2.60	.0481
1.4	2.25	33.0	2.20	.0562
1.6	2.00	9.6	——	.0641
1.8	1.95	8.4	1.97	.0719
2.0	1.85	0	1.85	.0800

TABLE 2

Na_2SO_4(2N) Av. T. 23° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.25	100.0*	4.70	——
.3	5.20	108.7	——	.0120
.6	5.30	106.3	——	.0241
.8	5.18	97.3	——	.0321
1.0	——	103.9	4.70	.0400
1.2	——	93.5	——	.0481
1.4	5.25	95.3	——	.0562
1.6	——	99.7	——	.0641
1.8	——	94.2	——	.0719
2.0	5.25	91.3	4.70	.0800

TABLE 3

H_3PO_4(3N/2) Av. T. 24° C.

cc.	Initial pH	% Growth	Final pH	N
0	6.85	100.0*	4.55	——
1	5.90	102.9	4.30	.0300
2	3.90	116.6	3.90	.0602
3	3.35	106.6	3.45	.0901
5	2.64	78.5	2.62	.1502
6	2.55	35.3	——	.1802
8	2.20	0	2.16	.2409
11	1.95	——	1.95	.3300
13	——	——	——	.3906
15	1.78	——	——	.4950

TABLE 4

NaH_2PO_4(3N/2) Av. T. 24° C.

cc.	Initial pH	% Growth	Final pH	N
0	6.85	100.0*	4.55	——
1	6.35	90.5	——	.0300
2	——	78.1	——	.0602
3	5.32	83.9	——	.0901
5	5.18	74.7	4.65	.1502
6	5.11	82.2	——	.1802
8	——	——	——	.2409
11	5.04	73.3	——	.3300
13	——	——	——	.3906
15	4.95	62.0	4.50	.4950

TABLE 5

HCOOH(N/1) Av. T. 22° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.20	100.0*	4.50	——
.2	4.88	99.0	4.45	.0040
.4	4.60	100.3	4.53	.0080
.6	4.37	108.2	4.51	.0120
.8	4.20	97.1	4.45	.0160
1.0	4.10	86.8	4.49	.0200
1.2	3.99	44.3	4.49	.0239
1.4	3.87	0	——	.0280
1.6	3.82	——	——	——

TABLE 6

HCOONa(N/1) Av. T. 22.1° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.80	100.0*	4.32	——
—	——	——	——	.0040
.4	——	94.3	——	.0080
.6	4.83	100.0	4.73	.0120
—	——	——	——	.0160
1.0	4.86	99.4	4.90	.0200
1.2	4.89	100.0	4.91	.0239
—	——	——	——	——
—	——	——	——	——

Experimental Data Showing the Influence of the Addition of Certain Acids and Their Sodium Salts upon the Growth of Sclerotinia cinerea

TABLE 7

CH_3COOH (N/1) Av. T. 22° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.20	100.0*	4.50	——
.2	4.96	102.7	4.47	.0040
.4	4.66	109.0	——	.0080
.6	4.65	90.1	4.53	.0120
.8	4.56	76.9	——	.0160
1.0	4.50	21.6	4.55	.0200
1.2	4.45	0	——	.0239
1.4	——	——	——	——

TABLE 8

CH_3COOH (N/1) Av. T. 24.6° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.29	100.0*	4.62	——
—	——	——	——	——
.4	4.84	94.9	4.59	.0080
—	——	——	——	——
.8	4.65	44.4	4.57	.0160
1.0	4.54	5.6	4.55	.0200
1.2	——	0	——	.0239
—	——	——	——	——

TABLE 9

CH_3COONa (N/1) Av. T. 22.1° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.80	100.0*	4.32	——
.4	5.00	101.5	4.65	.0080
.6	——	100.0	——	.0120
.8	——	87.9	——	.0160
1.0	5.20	100.0	5.04	.0200
1.2	——	——	——	.0239

TABLE 10

CH_3COOH (N/1) Av. T. 23.5° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.65	100.0*	——	——
—	——	——	——	——
—	——	——	——	——
.8	4.89	78.1	——	.0160
1.0	4.70	51.1	——	.0200
1.2	4.69	0	——	.0239

TABLE 11

C_3H_7COOH (N/10) Av. T. 22.1° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.80	100.0*	4.26	——
.5	4.76	61.1	4.30	.0010
1.0	4.70	61.1	4.37	.0020
1.5	4.65	53.1	4.40	.0030
2.0	4.61	10.6	4.55	.0040
2.5	4.5+	3.2	——	.0050
3.0	——	0	——	.0060

TABLE 12

C_3H_7COOH (N/1) Av. T. 23.3° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.20	100.0*	——	——
.2	4.91	47.3	4.70	.0040
.4	4.79	0	——	.0080

Experimental Data Showing the Influence of the Addition of Certain Acids and Their Sodium Salts upon the Growth of Sclerotinia cinerea

TABLE 13

C_3H_7COONa (N/10) Av. T. 24.6° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.70	100.0*	4.30	——
.5	4.74	46.7	4.15	.0010
1.0	——	——	——	.0020
1.5	4.79	14.2	4.45	.0030
2.0	4.81	4.4	4.68	.0040
2.5	4.83	3.6	4.77	.0050

TABLE 14

C_3H_7COOH (N/10) Av. T. 23.5° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.80	100.0*	——	——
.5	——	56.5	——	.0010
1.0	——	52.0	——	.0020
1.5	——	4.5	——	.0030
2.0	——	0	——	.0040

TABLE 15

C_3H_7COONa (N/10) Av. T. 23.5° C.

cc.	Initial pH	% Growth	Final pH	N
0	4.80	100.0*	——	——
.5	——	48.6	——	.0010
1.0	——	41.8	——	.0020
1.5	——	40.7	——	.0030
2.0	——	4.5	——	.0040
2.5	——	3.4	——	.0050

TABLE 16

$C_6H_4OHCOOH$ (N/100) Av. T. 24.6° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.10	100.0*	4.34	——
2	——	88.6	——	.0004
6	5.02	78.2	4.37	.0012
10	4.90	75.5	——	.0020
15	4.85	75.9	4.36	.0030
18	4.75	81.2	4.35	.0036
21	4.69	56.4	3.75	.0042
23	——	7.4	3.90	.0046
25	4.64	4.8	4.35	.0050

TABLE 17

$C_6H_4OHCOONa$ (N/100) Av. T. 24.6° C.

cc.	Initial pH	% Growth	Final pH	N
0	5.10	100.0*	4.34	——
6	——	66.0	4.40	.0012
15	5.09	74.5	4.50	.0030
21	——	78.2	4.48	.0042
25	5.08	72.9	4.40	.0050

* Mean weight of control, 0.805 g.

it lowered these as a rule. With phosphoric acid, the final acidity of those cultures whose original acidity was lower than pH 3.90 was raised, while those cultures whose original acidity was greater than pH 3.90 were either raised or lowered. With formic acid, the final pH of the culture medium varied from 4.45 to 4.51. The final pH values of the acetic-acid series varied with the series from pH 4.47 to pH 4.62. In the butyric-acid series, the final pH values of the culture medium were arranged in regular order as seen in table 11. With salicylic acid, the final pH values of the different culture media varied but were in the neighborhood of pH 4.35 as a rule.

It was extremely interesting to observe the changes that took place in the character of the mycelium as the culture medium became progressively more unfavorable for fungous growth. In most cases, as the medium became more acid there was a marked increase in the number of spores, and the mycelium, which was whitish and evenly covered the surface of the culture medium of the controls, became coated with brownish spores and irregularly distributed. As acidity increased, the amount of mycelium decreased and it formed small, more or less isolated colonies which were often raised and generally, although not always, tended to form a ring on the side of the flask. Finally, in those cultures which just permitted growth, only a few scattered colonies about the size of pin-heads were present. In certain cases (salicylic acid), the mycelium showed a tendency to wrinkle as the acidity of the medium was increased. With the fatty acids, the culture solutions became progressively redder toward the acid end of the series as a result of fungous growth. Those cultures in which *Sclerotinia cinerea* was unable to grow kept their original color.

The hydrogen-ion concentration of the first cultures which did not show growth depended upon the acids used. Thus the following values were secured: sulfuric acid, pH 1.85; phosphoric acid, pH 2.20 (although one small colony was observed at this concentration); formic acid, pH 3.87; acetic acid, pH 4.45; butyric acid, pH 4.5; salicylic acid, slightly above pH 4.64. These values with the fatty acids, as will be shown later, were dependent upon the initial acidity of the culture solution.

Tables 2, 4, 6, 9, 13, 15, and 17 give the data for the corresponding sodium salts of the acids. It was found that all the salts used gave good growth except sodium butyrate, but only in the case of sodium sulfate and sodium acetate did the growth ever exceed that of the control.

The original hydrogen-ion concentration was changed by fungous growth. In the case of sodium sulfate (table 2), it reached pH 4.70; with sodium dihydrogen phosphate, pH 4.50–4.65 (table 4). With sodium formate (table 6), sodium acetate (table 9), and sodium butyrate (table 13), the final pH values arranged themselves in a progressive series, and with sodium salicylate (table 17), the final pH values varied with the concentration.

Unfavorable conditions of the medium for fungous growth were shown by the increased amount of spore-production in the sulfate, phosphate,

butyrate, and salicylate cultures. In the case of the two latter salts, the mycelium showed a marked tendency to wrinkle as the salt concentration increased, and in the sodium butyrate cultures of higher salt concentrations it appeared much like the mycelium in the higher butyric-acid concentrations.

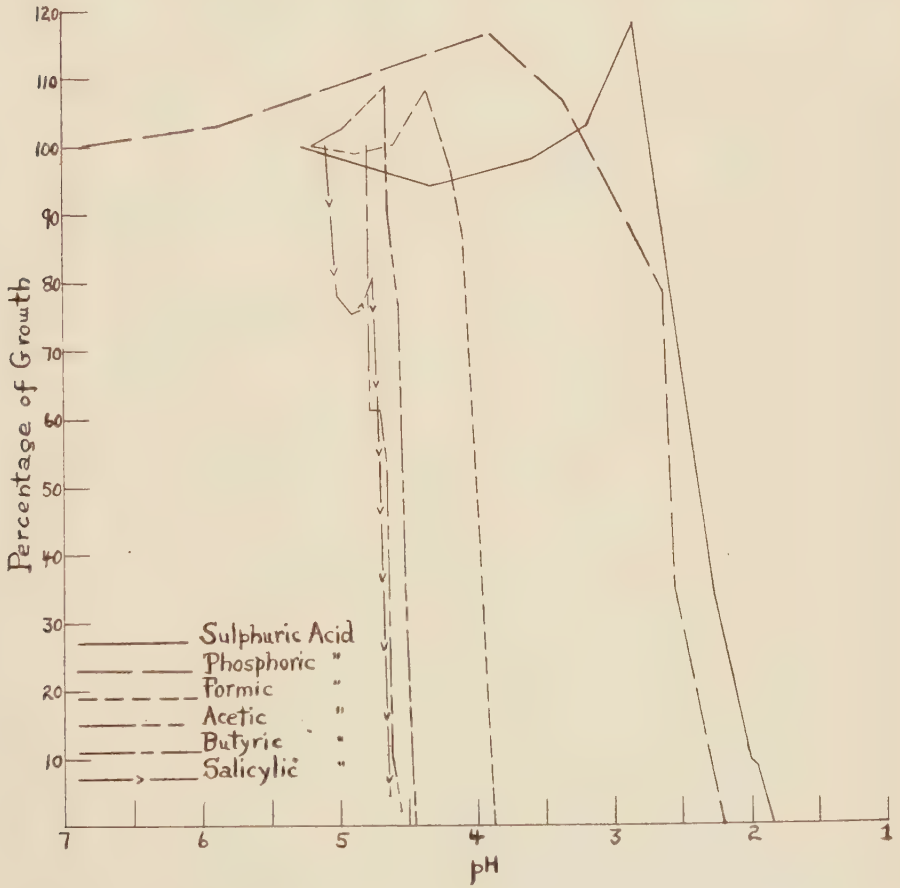
The percentages of relative growth values for the fungus in the sodium-salt cultures corresponding to the acid cultures in which the least amount of growth was obtained for a particular series were found to be as follows: sodium sulfate, 94.2 percent; sodium hydrogen phosphate, 82.2 percent; sodium formate, 100 percent; sodium acetate, 100 percent; sodium butyrate, 3.6 percent and 4.7 percent; and sodium salicylate, 72.9 percent. The toxic effect of sodium butyrate was marked; in both series run with this salt (tables 13, 15), 2.5 cc. of N/10 salt allowing only 3.4 percent–3.6 percent growth.

Discussion

A comparison of the order of toxicity of equinormal solutions of a series of acids should give some indication of the importance of the hydrogen ion as a factor in toxicity. If the hydrogen ion is the chief factor or sole factor of toxicity of the acids, then those acids which are more highly dissociated should be relatively more toxic. Owing to the widely different concentrations necessary under the conditions of these experiments, a strict comparison of percentage growth in equinormal solutions is impossible. For example, the solubility factor of salicylic acid made advisable the use of a higher dilution (N/100) of this acid. At the same time, the final volume of the culture medium in all culture flasks was limited to 50 cc., 25 cc. of which was basal nutrient solution. At N/32, sulfuric and phosphoric acids were practically equal in value (102 percent–103 percent) while at N/16, sulfuric acid gave 10 percent and phosphoric acid over 110 percent growth. At N/200, formic acid gave 99+ percent growth, acetic acid 105 percent in one series, butyric acid 3.2 percent–3.4 percent, and salicylic acid 4.8 percent. With the concentrations used greater than N/125, acetic acid becomes more toxic than formic acid. With butyric acid at N/800, the percentage growth was approximately between 50 percent and 60 percent, while with salicylic acid it was 78 percent; with butyric acid N/333, 53.1 percent in one case (table 11), and 4.5 percent (table 14) in another; with salicylic acid N/333, there was 75.9 percent growth. It may be seen from figure 3 that butyric acid is more toxic than salicylic acid at all the comparable concentrations used.

The order of toxicity found in these experiments for equinormal solutions is as follows: at N/20 and at higher concentrations, sulfuric > phosphoric; at N/200, butyric > salicylic > formic > acetic; with N/83 and higher concentrations, acetic > formic. The general order found when based on molecular concentration is butyric > salicylic > acetic > formic > sulfuric > phosphoric (text fig. 3). Clark (2) has shown that in equinormal

solutions acetic acid is much more toxic than sulfuric and hydrochloric acids to Botrytis and certain other fungi. Collett (4) found the general order for Paramoecium in equinormal solutions to be hydrochloric > salicylic > formic > acetic and butyric acids. Kahlenberg and True (9) in their work on Lupinus seedlings showed the order of toxicity for equinormal solutions to be hydrochloric > salicylic > formic > butyric. Bodine (1) gives the general order of toxicity for mosquito larva: salicylic > butyric > acetic. The results here reported differ from those of the three last-



TEXT FIG. 2. Percentage of growth of *S. cinerea* at various hydrogen-ion concentrations with various acids.

mentioned workers in that sulfuric acid is less toxic than the fatty acids, and that butyric acid is more toxic than either salicylic or formic. The above-named writers, except Clark, used very weakly buffered solutions where additions of equal amounts of different acids must have produced far different degrees of acidity. In these experiments, where a highly buffered

medium has been used, the buffer action of the solution would reduce the relative differences in the actual acidity produced by highly and less highly dissociated acids. Hence it is probable that the buffer action of the solution is a factor which has changed the relative position of the mineral acids in the series. Increasing the length of exposure to mineral acids because of their slow penetration into a cell should tend, if it causes any change, to render the acid relatively more toxic than the acids which enter more readily. If this assumption is true, it would seem to indicate that the toxicity of the mineral acids has not been exaggerated in these experiments where a longer time limit than in the investigation of others was chosen as a criterion.

The results show that the hydrogen ion is not the only factor or the chief factor to be considered in the toxicity of salicylic and the fatty acids to *Sclerotinia cinerea*, since the order of toxicity does not parallel the order of dissociation for these acids.

It is of interest to notice that the relative toxicities of salicylic and the fatty acids in equinormal solutions for *S. cinerea* are in accord with the order that would be expected from the standpoint that the more highly developed the non-polar part of the molecule, the more readily primary penetration occurs (7). The mineral acids used also are in the position in the series that we should expect when we view these acids as only entering the cell as a result of the injury to the cell by the H ions, higher concentrations therefore being necessary to produce toxic action.

Another source of evidence of the toxicity of the hydrogen ion is a comparison of the percentage of growths of *Sclerotinia cinerea* obtained with the acids used at the same hydrogen-ion concentration. If the hydrogen ion is the only toxic factor, then it is clear that at the same pH the acids should be equally toxic, and the percentage growths should be about equal. It may easily be seen from figure 2 that this is not the case. At pH 4.0, there is about 45 percent of the growth of the control with formic acid, 96 percent with sulfuric acid, and 116 percent with phosphoric acid; and at pH 4.70, with salicylic acid 59 percent, with butyric acid 61 percent, with sulfuric acid 96 percent, with formic acid 100 percent, with acetic acid 108 percent, and with phosphoric acid 111 percent. As may be seen, the curves for the percentage growth of the different acids cross each other at certain points (fig. 2). The curves for sulfuric and phosphoric acids are alike in many respects—approximately 80 percent growth being obtained at pH 2.6 and pH 2.68 respectively. The general order of toxicity at pH 4.70 is salicylic > butyric > sulfuric > formic > acetic > phosphoric, while with a little higher hydrogen-ion concentration (pH 4.5), acetic acid is far more toxic than sulfuric acid, and at pH 4.4 and above formic acid is also more toxic.

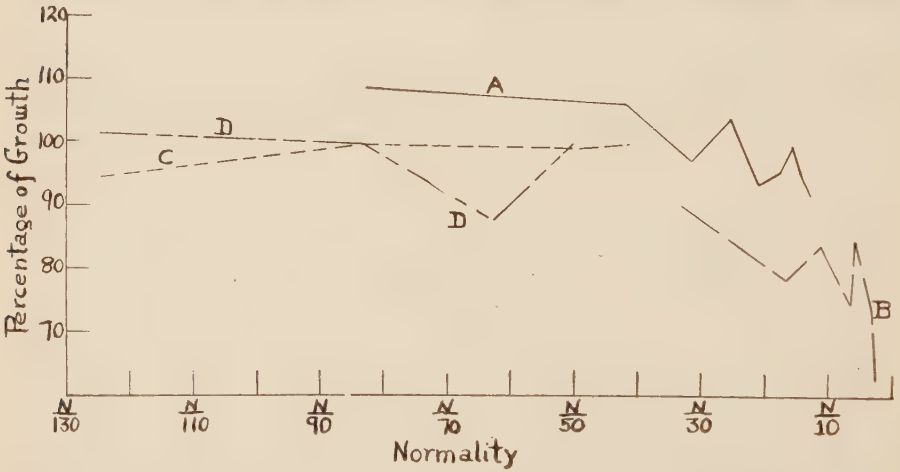
It may be seen that in the comparison of solutions of equal pH, salicylic and the fatty acids keep their position as being more toxic than the inorganic acids. Collett (4), in describing her experiments on Paramoecium, states that in solutions of equal pH acetic and butyric acids become equal in toxic-

ity to the cyclic acids and that the dibasic and hydroxy acids together with hydrochloric acid become least toxic of all. The fact that fatty acids are more effective physiologically than hydrochloric acid of the same H-ion concentration has been observed by certain workers. For example, Loeb (11) found in his experiments on membrane-formation in sea-urchin eggs that CO_2 and the fatty acids were efficient and that strong mineral acids were inefficient. He explained the phenomena on the basis of the greater diffusibility of the non-dissociated acid molecule through the cell wall. It is striking to note that the order of toxicity of the acids in the complex nutrient solution used in these experiments is of the same order as that found in the above-mentioned investigations. It is thus safe to assume that these results indicate the toxicity of the acid used rather than that of any uncertain factor which may have been introduced by the complex solution. Again, if the effect on growth is proportional to the ability of the acid to penetrate the cell and to produce some physiological upset, we have indirect evidence that we should expect this order of toxicity from the work on permeability (7).

TEXT FIG. 3. Percentage of growth of *S. cinerea* at various normalities of the acids used.

The very fact that with salicylic and the fatty acids no growth occurs under the conditions of the experiment at a higher hydrogen-ion concentration than pH 3.99 shows that the hydrogen ion can not be the chief factor in toxicity, whereas in the case of sulfuric acid growth was found at pH 1.95 and with phosphoric acid at pH 2.2. Furthermore, acetic-acid cultures containing only the nutrient solution with 1 cc. of normal acetic acid and

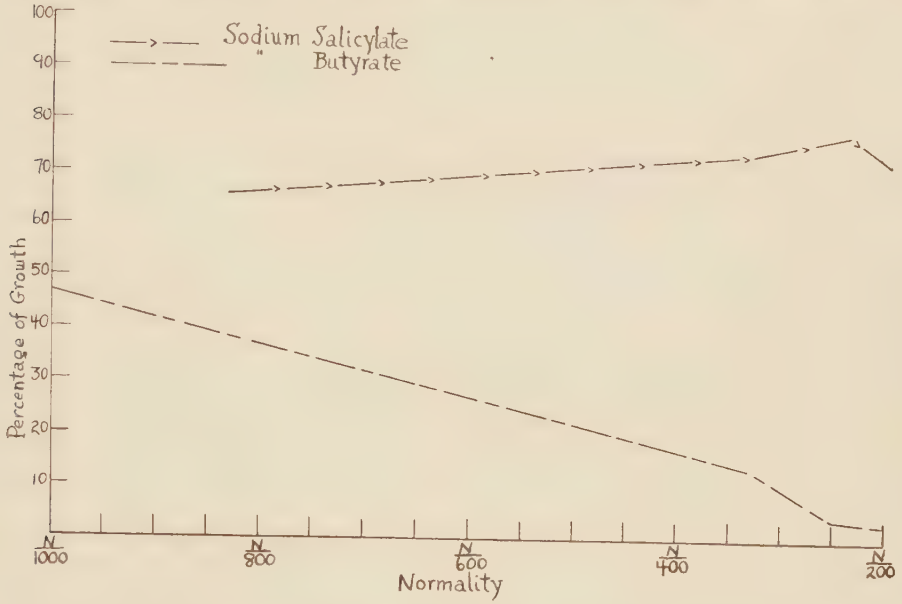
with no sodium hydroxid added failed to give growth although the pH was only 3.85. Likewise, another series of acetic-acid cultures, in which the acidity of the nutrient solution was lowered by the addition of sodium hydroxid to pH 5.65 (table 10), failed to give growth at pH 4.69 with 1.2 cc. normal acetic acid added. Butyric-acid cultures likewise, when the original nutrient solution was at pH 4.80 showed a hydrogen-ion concentration at growth limit of about pH 4.5, while, when the original hydrogen-ion concentration of the nutrient solution was pH 5.2, growth ceased at pH 4.79. In the cases of salicylic and the fatty acids used, it seems that the original pH of the medium and the amount of the acid added, and not the hydrogen-ion concentration alone, determined the limits of growth of *Sclerotinia cinerea*.



TEXT FIG. 4. Percentage of growth of *S. cinerea* at various normalities of (*A*) sodium sulfate, (*B*) sodium dihydrogen phosphate, (*C*) sodium formate, (*D*) sodium acetate.

Since it is evident that, in the cases of the fatty acids used and of salicylic acid, the hydrogen ion is not the sole factor, it is obvious that the toxicity is due to other causes. It is impossible to show directly that either the anion or the undissociated molecule of the added acids is the toxic agent until the combinations formed in the complex highly buffered nutrient solution by the added acids and their percentage dissociation can be determined. However, a comparison of the salt solutions in equinormal concentrations with their acids shows certain tendencies. Figures 4 and 5 show the kind of curves obtained for the various sodium salts used. Series were run for each salt which duplicated the steps of the series of the corresponding acids. By a comparison of the percentage growth curves, it may be seen that the salts of the acids are in general far less toxic than the acids themselves. Sodium butyrate alone shows a tendency to approach the toxicity of its acid. In one series (table 13), for example, sodium butyrate N/333 gave a growth of 14.2 percent, while butyric acid N/333 gave a value of 53.1 per-

cent. In the same series, sodium butyrate N/250 gave 4.4 percent growth while butyric acid N/250 allowed 10.6 percent. In another series, sodium butyrate N/333 gave a growth value of 40.7 percent and at N/250 only 4.5 percent. Butyric acid in the latter series at N/333 gave a value of 4.5 percent and at N/250 no growth. While it is not possible to state the percentage dissociation of the salt solutions, it is known that there is a



TEXT FIG. 5. Percentage of growth of *Sclerotinia cinerea* at various normalities of sodium butyrate and sodium salicylate.

tendency for the salts of the acids to dissociate more highly than the corresponding acids. Since we know that the sodium cation is practically harmless, and if we assume higher dissociation of the salt than of the butyric acid, then it appears that the toxicity of butyric acid is in large measure due to the anion and the undissociated molecule. However, in the case of butyric acid, the relatively greater toxicity of the anion is apparent when compared with formic, acetic, and salicylic acids.

In all other cases with the higher concentrations used, the salt is much less toxic than the corresponding acid. Sodium dihydrogen phosphate gives between 70 percent and 80 percent growth at the limiting concentration for phosphoric acid, while sodium sulfate gives 94.2 percent. It appears that in the case of these mineral acids, since ionization is high, the main toxicity factor is the hydrogen ion. This agrees with the work of True (15) and Winslow and Lochridge (17).

With respect to formic, acetic, and salicylic acids, if no other toxic compounds are formed and if the salt dissociates far more than its acid in the

nutrient solution used, then the undissociated molecule may be suspected as the principal toxic factor. The anion appears to be relatively nontoxic. This is in accord with the results of Collett (4), Kahlenberg and True (9), True (15), and Winslow and Lochridge (17).

Summary

1. The addition of sodium hydroxid is practically harmless in changing the pH from 3.8 or 4.0 to 5.2 or slightly higher.

2. A slight amount of acidity is beneficial for growth, the best results with sulfuric and phosphoric acids being obtained between pH 2.85 and pH 3.9.

3. There is a fairly narrow zone on the acid side which limits growth for each acid used—the percentage growths falling in an almost perpendicular line (fig. 2).

4. The general order of toxicity for solutions under the conditions of these experiments at pH 4.70 is salicylic > butyric > sulfuric > formic > acetic > phosphoric, while at pH 4.50, acetic is more toxic than sulfuric, and at pH 4.4 formic is also more toxic than sulfuric.

5. A comparison of the toxicity of the acids on a basis of normality gives the general order: butyric > salicylic > acetic > formic > sulfuric > phosphoric. This is the order that would be expected from the comparative ease of penetration of the acids into the living cell as has been shown in other investigations.

6. There is indication that the anion of butyric acid may be relatively toxic.

7. The toxicity of the fatty acids used and of salicylic acid is probably due chiefly to the undissociated molecules, with the hydrogen ion playing a secondary rôle.

8. On the other hand, the hydrogen ion is the principal factor of toxicity in the case of the mineral acids used.

In conclusion, these results show that the hydrogen ion is not always the chief factor of toxicity in the effect of various acids upon the germination and growth of fungous spores.

The author wishes to express to Dr. Charles H. Arndt his appreciation and thanks for many valuable suggestions and constant interest throughout the progress of the work.

Department of Botany,
University of Pennsylvania

LITERATURE CITED

1. **Bodine, J. H.** A note on the toxicity of acids for mosquito larvae. Biol. Bull. 45: 149. 1923.

2. **Clark, J. F.** On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. 28: 289–327, 378–404. 1899.

3. **Clark, W. M.** The determination of hydrogen ions. Baltimore, 1923.
4. **Collett, M. E.** Toxicity of acids to ciliate infusoria. Jour. Exp. Zool. **29**: 443–472. 1919.
——. Toxicity of acids to infusoria II. The rôle of molecule and of ions. Jour. Exp. Zool. **34**: 67. 1921.
5. **Dunn, M. S.** The microloop. A rapid method for isolating single spores. Phytopath. **14**: 338. 1924.
6. **Fred, E. B., and Loomis, N. E.** Influence of hydrogen-ion concentration of medium on the reproduction of alfalfa bacteria. Jour. Bact. **2**: 629–633. 1917.
7. **Jacobs, M. H.** General cytology, pp. 99–164. Chicago, 1924.
8. **Johnson, H. W.** Relationships between hydrogen ion, hydroxyl ion, and salt concentrations and the growth of seven soil molds. Iowa State Coll. Agr. Exp. Sta. Res. Bull. 76. 1923.
9. **Kahlenberg, L., and True, R. H.** On the toxic action of dissolved salts and their electrolytic dissociation. Bot. Gaz. **22**: 81–124. 1896.
10. **Krönig, B., and Paul, T.** Die chemischen Grundlagen der Lehre von der Giftwirkung und Disinfection. Zeitschr. Hygiene Infectionskrankh. **25**: 1–112. 1897.
11. **Loeb, J.** Artificial parthenogenesis and fertilization. Chicago, 1913.
——. Sodium chloride and selective diffusion in living organisms. Jour. Gen. Physiol. **5**: 231–254. 1922.
12. **Morgan, J. F., and Gruzit, O. M.** Reaction of the soil solution as an index of biological changes in the soil. Jour. Bact. **1**: 97. 1916.
13. **Norton, J. F., and Hsu, P. H.** The physical chemistry of disinfection. Jour. Infect. Dis. **18**: 180–194. 1916.
14. **Taylor, K.** Specificity in antiseptics. Lancet **1**: 294–296. 1917.
15. **True, R. H.** The toxic action of a series of acids and of their sodium salts on *Lupinus albus*. Amer. Jour. Sci. **9**: 183–192. 1900.
16. **Webb, R. W.** Studies in the physiology of the fungi X. Germination of the spores of certain fungi in relation to hydrogen ion concentration. Ann. Mo. Bot. Gard. **6**: 201–222. 1919.
17. **Winslow, C.-E. A., and Lochridge, E. E.** The toxic effect of certain acids upon typhoid and colon bacilli in relation to the degree of their dissociation. Jour. Infect. Dis. **3**: 547–571. 1906.
18. **Wolf, C. G. L., and Harris, J. E. G.** Contributions to the biochemistry of pathogenic anaerobes III. The effect of acids on the growth of *Bacillus Welchii* (*B. perfringens*) and *Bacillus sporogenes* (Metchnikoff). Biochem. Jour. **11**: 213–245. 1917.